Topography of Proteoglycan and Glycosaminoglycan Free Chain Expression in 3T3 Fibroblasts and Human Keratinocytes

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Synthesis of heparan sulfate–free chains by human keratinocytes is upregulated during terminal differentiation. The cellular location of this product class and the significance of the differentiation effect are unknown. Differential plasma membrane shearing with cationized colloidal silica was used to evaluate the compartmentalization of the heparan and chondroitin sulfate free chains and their respective proteoglycans in 3T3 fibroblasts and human keratinocytes. The method exploits the topologic segregation of plasma membranes of adherent cells into ventral, dorsal, and intracellular domains and the selective binding of the silica to the dorsal membranes, which by shearing can be separated from ventral membranes adherent to the substratum. Analysis of membrane preparations from sheared cells that had been prelabeled with [^{35}S]-sulfate revealed the proteoglycans to be predominantly ventral, at which location a matrix binding function could be accommodated. Proteoglycans were also recovered from dorsal and intracellular membranes, suggesting active trafficking between intra- and extra-cellular sites. In contrast, the major fraction of heparan and chondroitin sulfate free chains was either cytosolic or associated with intracellular membranes, with the remaining ~20% segregated to dorsal and ventral membranes. These results suggest different cellular functions for the proteoglycans and glycosaminoglycan free chains. The partial localization of the free chains to peripheral membranes is compatible with our prior hypothesis that they arise by processing of precursor proteoglycans on cell surfaces. Following this origin, the free glycosaminoglycan polymers could be available to bind ligands such as cytokines prior to transport to intracellular sites of action. *J Invest Dermatol 99:386–389, 1992*

Amid the range of biologic activities that have been attributed to the proteoglycans, two general categories of function emerge from the broader perspective. In extracellular matrices, proteoglycans participate in the assembly of other matrix components. On cell surfaces, they are likely to be involved in the regulation of cell growth [1]. Whatever the precise functions within these general categories, the carbohydrate side chains that are attached to the core proteins of the proteoglycans presumably mediate some of the binding interactions with ligands and other macromolecules. This biologic reactivity derives from the highly polyanionic nature and the structural diversity of the complex heterodimeric polymers, termed glycosaminoglycans (GAG), that constitute the side chains. The molecular heterogeneity that is inherent in the GAG has evolved the furthest in the case of the heparan sulfates, where such complexity likely represents mechanisms for the cellular regulation of the functional interactions of GAG with other molecules. Interest in the proteoglycans has recently grown due to experimental evidence suggesting a role for heparan sulfates in regulating the mitogenic signaling of the fibroblast growth factor family of cytokines [2].

Although sulfated GAG are conventionally considered to function in covalent linkage to the core proteins of proteoglycans [1], GAG in apparent free polymeric form have been recovered from a diversity of cell types under a variety of conditions (reviewed in [3]). From their resistance to alkaline reduction and relatively low Mr, these GAG free chains are distinct from the customary proteoglycan classes [4]. Other investigators have proposed that they represent intracellular lysosomal degradation products of proteoglycans based in part on their smaller size and inaccessibility to mild trypsinization of intact cells [5]. A precursor-degradative product relationship, however, may be an overly simplified model inadequate to accommodate all of the data. Pharmacologic inhibition of lysosomal function, for example, does not block the appearance of the free chains as it should if they were to arise from lysosomal processing [6]. Furthermore, a portion of the GAG free chains, in addition to most of the proteoglycans, has proved accessible to digestion in situ with GAG degrading enzymes under conditions in which cell viability is not materially altered [3,6]. Consequently, we have hypothesized that GAG free chains arise by processing from proteoglycans on cell surfaces, a mechanism that would provide a potential source of free polymer available for binding of ligands prior to transport to intracellular sites of action [4].

One consideration bearing on the localization of cellular macromolecules is that cultured, adherent cells are topologically polarized, such that a ventral cell membrane domain approximates the substratum and a dorsal domain contacts the fluid media. In addition, there is a large intracellular pool of plasma membranes in equilibrium with both domains [7]. Mason and Jacobson [7] ex-

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Abbreviations:
GAG: glycosaminoglycan
HEK: human epidermal keratinocyte

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ploited the selective binding of cationic colloidal silica to the dorsal surface of adherent cells to separate the compartments. With cell lysis, the silica-coated dorsal membranes can be separated on the basis of density from intracellular membrane components by centrifugation, whereas the ventral domain remains adherent to the substratum. Information on the localization of cellular proteoglycans and GAG free chains to these domains could provide insight into the respective physiologic functions of the two categories of molecules. In the present report, differential cell membrane separation achieved with the cell-shearing method was used to probe the compartmentalization of proteoglycans and GAG free chains in murine fibroblasts and human epidermal keratinocytes (HEK).

MATERIALS AND METHODS

Materials Chromatographic media were from Pharmacia (Piscataway, NJ) and Bio-Rad (Richmond, CA). Tissue-culture procedures utilized Corning flasks and 6-well plates (Corning, NY), Eagle's modified medium (Flow Laboratories, McLean, VA), and fetal bovine serum (Irvine Laboratories, Santa Anna, CA). GAG markers were bovine lung heparan sulfate [8] and chondroitin 6-sulfate and human umbilical hyaluronan from Sigma (St. Louis, MO). Du Pont-New England Nuclear (Boston, MA) supplied $Na_2[^{35}S]O_4$ (370 mCi/mM). A multifunctional Flavobacterial "heparinase," which degrades all GAG except keratan sulfate and lacks protease activity [9,10], was prepared as indicated [11]. Colloidal cationic silica was a generous gift from Mason and Jacobson [7].

Cell Lines BALB/c 3T3 clone A31 fibroblasts, purchased from the American Type Culture Collection (Rockville, MD), were cultured in medium supplemented with 10% fetal bovine serum. HEK were purchased from Clonetics (San Diego, CA) and cultured with KGM medium according to supplied recommendations.

Labeling and Extraction of Cellular Glycoconjugates 3T3 fibroblasts in 75-cm² flasks were metabolically labeled for 48 h under exponential growth (~75% confluence) with [^{35}S]-sulfate (90 μCi/ml) in complete growth medium. The HEK were similarly labeled, but in KGM medium without serum. After washing of the cultures, labeled products associated with the cells or various membrane preparations (see below) were extracted for 30 min at 37°C with 1% Triton X-100 and 4 M urea in 25 mM Tris-HCl, pH 7.5, containing 10 mM N-ethylmaleimide, 100 mM epsilon-aminocaproic acid, 10 mM EDTA, and 5 mM benzamidine HCl.

Analytical Methodology The cell and membrane preparations were eluted from 110 × 1 cm columns of Sepharose CL-4B, with 0.35 m NaCl, 50 mM Tris-HCl, 0.1% sodium dodecyl sulfate, and protease inhibitors as eluent. Identification of the proteoglycan and GAG free chain content of the fractions was obtained by the presence or absence of a K_{av} shift following reduction with alkaline borohydride (0.5 M sodium hydroxide and 0.005 M sodium borohydride) for 24 h at 4°C. Other analytical methods were as specified [3,12].

Procedure for Enzymatic Localization of Glycoconjugates The previously described protocol was followed for the digestion of intact cell layers with GAG-degrading enzymes [3,4,6]. In brief, metabolically prelabeled cultures were treated with Flavobacterial heparinase (1 mg/ml in phosphate-buffered saline) for 30 min at 4°C. After washing, material remaining associated with the cells was extracted standardly with Triton X-100 and protease inhibitors, as described above, and analyzed in parallel with that extracted from control cultures treated with buffer alone.

Shearing Method In separate experiments, BALB/c 3T3 fibroblasts and HEK were cultured in 6-well plates for biochemical analysis and on Falcon Cyclopore membrane inserts for histology and autoradiography. The cultures were prelabeled with [^{35}S]-sulfate for 2 d, then one-half of them were treated with multifunctional heparinase (1 mg/ml) and the other half with phosphate-buffered saline for 1 h. Cultures not to be sheared were harvested at this time for biochemical analysis and for histology and autoradiography. The remaining cultures were sheared according to Mason and Jacobson [7]. In brief, colloidal silica in 20 mM 4-morpholineethanesulfonic acid-buffered saline was added to the cultures, followed sequentially by polyacrylic acid (1 mg/ml) for cross linking and hypotonic imidazole (2.5 mM) buffer in 50% (v/v) glycerol with 100 microgram/ml heparan sulfate, as carrier, for shearing. The buffer was forced over the surfaces of the cultures with a pipette to facilitate shearing of the osmotically swollen cells. Material thus released from the monolayers was centrifuged to separate dorsal membranes adherent to the high-density silica from the supernatant fractions. These preparations, along with the ventral membranes remaining attached to the wells, were solubilized with detergent, as before, for biochemical analysis. The ventral membranes on the inserts were processed for autoradiography by standard methods.

Table I. Shearing Studies with 3T3 Cells

Condition	Compartment	Product (10^{-3} × [^{35}S]-Sulfate dpm)[a] Proteoglycan	Free Chain
No heparinase	Ventral	6.9	2.2
	Dorsal	5.1	1.6
	Supernate	10.5	16.9
	Whole cell[b]	30.5	35.2
Heparinase	Ventral	1.1	1.2
	Dorsal	0.9	1.3
	Supernate	2.3	16.3
	Whole cell[b]	3.3	26.6

[a] For the "no heparinase" condition, the ventral, dorsal, and supernate fractions of 3T3 cells collectively represented 74% and 59% recovery of proteoglycans and GAG free chains by reference to nonsheared cultures.
[b] Separate control cultures not sheared.

RESULTS

Tables I and II contain the data from the separate experiments with the 3T3 fibroblasts and HEK. For the purposes of these analyses, the chondroitin and heparan sulfate products have been combined for the categories of the proteoglycans and the GAG free chains because previous studies have suggested minimal differences in their compartmentalization [3,6]. Following heparinase treatment of intact cells that were not sheared, 89% and 82% of 3T3 fibroblast and HEK proteoglycans were respectively degraded by the enzyme, by reference to buffer-treated, nonsheared cultures, indicating their accessibility on the outer leaflet of the plasma membrane. The values for chondroitin and heparan sulfate free chain digestion were 24% for the 3T3 cells and 32% for the HEK products. As a reference to indicate the potential degree of experimental variability, 81% and 65% of the proteoglycans and 9% and 36% of the free chains from

Table II. Shearing Studies with HEK Cells

Condition	Compartment	Product (10^{-3} × [^{35}S]-Sulfate dpm)[a] Proteoglycan	Free Chain
No heparinase	Ventral	14.0	26.0
	Dorsal	1.8	10.0
	Supernate	2.0	140
	Whole cell[b]	27.0	179
Heparinase	Ventral	5.0	14.0
	Dorsal	0.2	11.0
	Supernate	1.1	87.0
	Whole cell[b]	4.9	122

[a] For the "no heparinase" condition, the ventral, dorsal, and supernate fractions of HEK collectively represented 66% and 98% recovery of proteoglycans and GAG free chains by reference to nonsheared cultures.
[b] Separate control cultures not sheared.

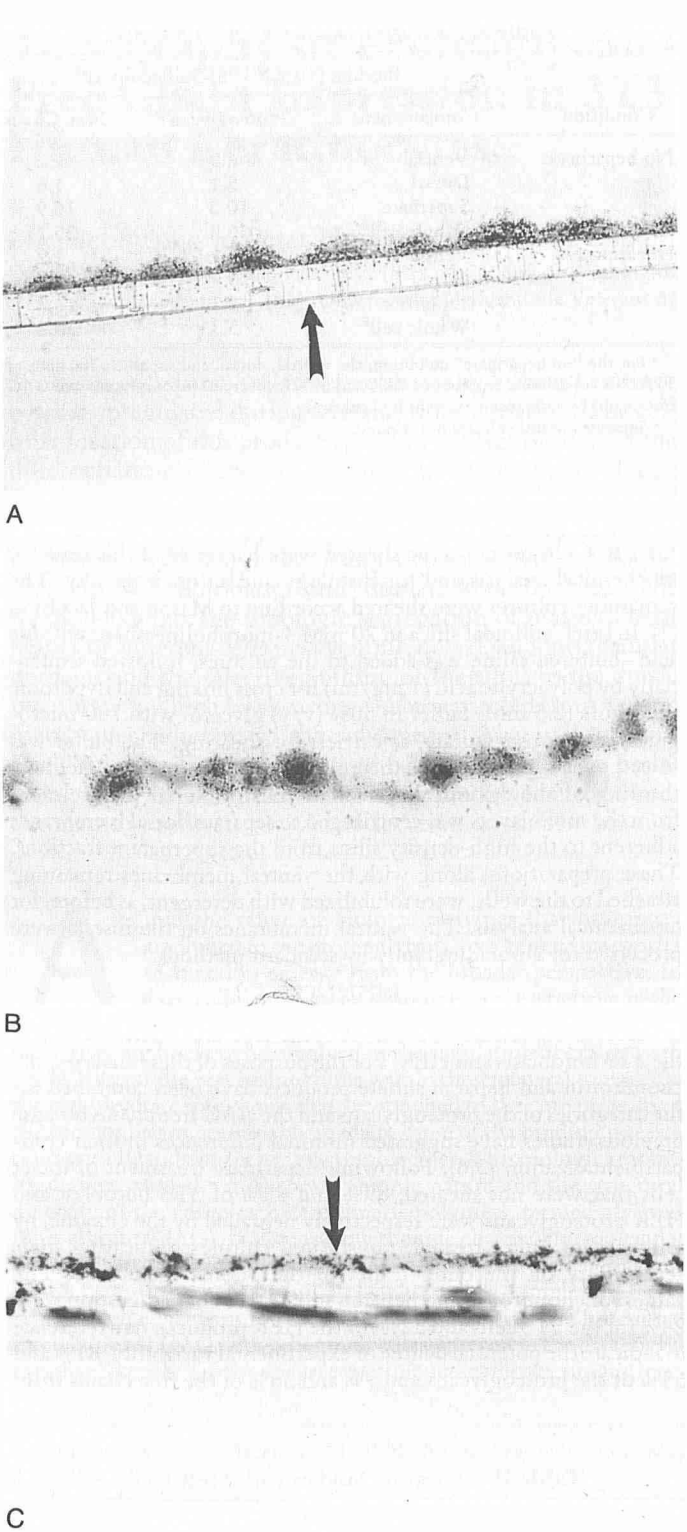

Figure 1. Autoradiographs of HEK, labeled for glycosaminoglycan content, with and without differential membrane shearing. HEK were metabolically labeled with [^{35}S]-sulfate, treated with either buffer *(A)* or with multifunctional heparinase *(B)*, and processed by autoradiography for 48 h. The *arrow* in *A* indicates the Cyclopore membrane to which the HEK are adherent in these vertical sections. Heparinase treatment *(B)* resulted in detachment of the cells from the Cyclopore membrane and reduced cell-cell adhesion. Separate cultures not treated with heparinase were sheared with the cationized colloidal silica, and autoradiographs were obtained from the membranes remaining attached to the substratum *(C)*. The *arrow* in this photomicrograph indicates the ventral membrane remnants, adherent to the Cyclopore substratum, that are decorated with the silver grains from the labeled product. (Hematoxylin and eosin counterstains; original magnifications for *A* and *B* are ×400; for *C*, ×600.)

the 3T3 fibroblasts and HEK, respectively, appear to be enzyme accessible when the buffer and heparinase treated sheared cultures are compared (Tables I and II).

For purposes of illustration, the relative compartmental distributions of the proteoglycans and free chains as revealed by shearing were calculated on the basis of total organic isotope recovery within cultures (i.e., not by reference to the nonsheared cultures) (Tables I and II). Following shearing, 31% of 3T3, and 79% of HEK, proteoglycans remained within the ventral domain with the rest appearing in the supernatant and dorsal preparations in a ratio of more than 1:1. Thus, much of the nonventral proteoglycans was seemingly not anchored to the dorsal membrane. For the free chain products of 3T3 cells, 11% was ventral, 8% was dorsal, and 82% was in the supernatant compartment after shearing. Values for HEK were 15% ventral, 6% dorsal, and 80% supernate. The 48–78% reduction in proteoglycans of the supernatant fraction following heparinase treatment (as compared with buffer-treated cultures; Tables I and II) could be an effect of reciprocal trafficking pathways of proteoglycans between intracellular and cell-surface compartments, such that material transported to the surface would be removed from the circuitry by heparinase in the culture milieu.

The autoradiographs showed circumferential grains around control HEK (Fig 1*A*) and 3T3 cells. A general reduction in labeling intensity was evident following heparinase digestion (Fig 1*B*). Resolution was not sufficient to further discriminate patterns of product distribution in treated or control cultures. Heparinase treatment, however, resulted in detachment of the cells from the substratum and reduced intercellular adhesion (Fig 1*B*), effects that are in general accord with the function of proteoglycans in cell-matrix interactions [10]. With shearing, ventral cell membranes were clearly evident as intact sheets on the Cyclopore substrate, and decorating these were grains of labeled product (Fig 1*C*). Thus, efficient membrane domain separation was achieved with the colloidal silica.

DISCUSSION

Our prior studies have emphasized the phenotypic diversity in proteoglycan synthesis between cell types. The diversity includes variations in relative mass of the proteoglycans and their GAG side chains, in the distribution of metabolic isotopic labeling among the major classes of GAG, and in content of GAG free chains [3,4,9]. HEK are phenotypically distinctive, in that the chondroitin and heparan sulfate proteoglycans are of relatively low average Mr (50–90 kDa), and the major fraction (~60–70%) of [^{35}S]-sulfate labeling is in apparent free chain form of mass ~7–28 kDa [4,13] (see also [14]). The cellular content of heparan sulfate free chains increases substantially with terminal differentiation of HEK [13], concurrently with an overall change in structure of the heparan sulfates [15].

Regardless of the diversity that is evident, one feature that is common among cells is that sulfated GAG free chains are partly expressed on the cell surface [3,4,6]. There have been differences, however, in the proportion of these products from different cell types that are accessible to enzymatic digestion on the external surface of the plasma membrane [4,13]. Compared with 3T3 fibroblasts, HEK in past studies have expressed pericellularly a variable fraction, which in part could reflect signficant experimental variation in the degree to which GAG lyases digest the HEK products in situ [4,13], or alternatively an effect related to presently uncharacterized growth conditions.

Because of the apparent differences between 3T3 fibroblasts and HEK in the surface expression of GAG free chains, as well as the potential interexperimental variability inherent to the past enzymatic digestion studies [4,13], we probed the localization of proteoglycans and GAG free chains by the use of differential shearing with cationized colloidal silica. The segregation of proteoheparan sulfates to the ventral domain of endothelial cell membranes has been characterized by this method [16]. Our results with the two distinct cell types under study were similar in certain respects, and these data permit some general conclusions. The majority of the HEK proteoglycans are in the ventral domain of the plasma membrane, in con-

tact with the culture substratum, at which location they would be appropriately situated to bind matrix molecules. Indeed, heparinase digestion of HEK resulted in reduced cell-cell and -matrix adhesion (Fig 1*B*). Less of the respective fibroblast proteoglycans, however, were so distributed. For both cell types, the remaining proteoglycans were partially dorsal, but a larger fraction was in the supernatant, representing either free cytosolic product or that associated with the intracellular pool of membranes. Either location could reflect a compartment in equilibrium with the peripheral membranes. Localization for the GAG free chains was different. Although the major fraction was in the supernate, ~20% of this product was nonetheless distributed to the combined ventral and dorsal domains for both cell types. This result is consistent with a transient peripheral membrane phase. The differences in localization for the major proteoglycans and the GAG free chains could reflect distinct functions.

The most parsimonious interpretation of all our data, which in addition to the present shearing studies includes differences between cell types and interexperimental variations in lyase accessibility [3,4,13], is that the free chains are generated and transiently expressed on cell surfaces but are then routed to intracellular sites of function. The fact that ~20% of the GAG free chains were recovered in the combined ventral and dorsal membrane domains after cell shearing is compatible with our prior hypothesis that these products are generated on cell surfaces. Differences in surface expression as revealed by GAG lyase accessibility could, according to this paradigm, reflect cell-type or growth-related differences in transit times between compartments. The finding of endoglycosidase activity in hepatocyte plasma membranes [17] supports the argument of cell-surface origin. The presence of heparan sulfate chains in hepatocyte nuclei [18] may, therefore, represent the ultimate functional targeting of free chains following their generation on cell surfaces. This transport could occur in conjunction with bound ligands such as growth factors.

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